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(71) Applicant *(for all designated States except US)*: THE WALTER AND ELIZA HALL INSTITUTE OF MEDICAL RESEARCH [AU/AU]; Royal Parade, Parkville, VIC 3052 (AU).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)* : LANGFORD, Christopher, John [AU/AU]; 803 Park Street, Brunswick West, VIC 3055 (AU). EDWARDS, Stirling, John [AU/AU]; 25 Henry Street, Eltham, VIC 3095 (AU).

(74) Agents: SLATTERY, John, Michael et al.; Davies & Collison, 1 Little Collins Street, Melbourne, VIC 3000 (AU).

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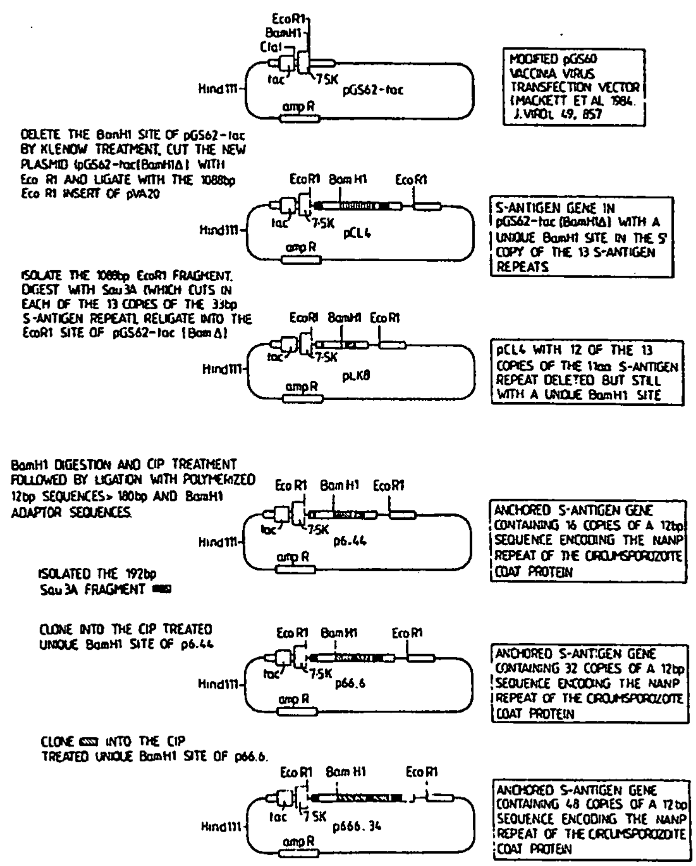
(54) Title: RECOMBINANT VIRUS

(57) Abstract

A recombinant virus, such as recombinant vaccinia virus, is characterised in that it includes a coding sequence for a hybrid polypeptide which comprises at least one immunogenic polypeptide segment which is foreign to the virus or virus infected cells in association with a surface or membrane-associated polypeptide segment to locate the hybrid polypeptide on or at the surface of virus infected cells.



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"RECOMBINANT VIRUS"

This invention relates to a recombinant virus, and in particular it relates to a recombinant vaccinia virus which has been modified to optimize the immunogenicity of foreign immunogenic polypeptides expressed thereby.

The term "recombinant virus" as used throughout this specification denotes infective virus which has been genetically modified by incorporation of foreign genes or genetic material into the virus genome. The modified virus then expresses the foreign gene in the form of a "foreign" polypeptide on infection of a cell by the recombinant virus. The term "recombinant vaccinia virus" has a corresponding meaning.

Since the development of methods for the expression of foreign genes in infective vaccinia virus (Mackett et al, 1982; Panicali and Paoletti, 1982) live recombinant vaccinia viruses have been shown to be of great potential in immunizing animals against infection with other more harmful viruses. This has been achieved by isolating the gene for target antigens of host protective immune responses and integrating them, under the control of vaccinia virus promoter elements, into

the vaccinia virus genome. In many respects the foreign viral antigen which is now expressed by the vaccinia virus is in a near normal situation and its processing, modification, transport and final localization on the surface of the infected cell may be very similar to that in a normal infection. It is therefore not surprising that when the herpes simplex glycoprotein D (Paoletti et al, 1984; Cremer et al, 1985), hepatitis B surface antigen (Smith et al, 1983; Moss et al, 1984), vesicular stomatitis virus glycoprotein G, and influenza virus hemagglutinin (Smith et al, 1983; Panicali et al, 1983) genes are inserted into recombinant vaccinia virus, live recombinant viruses can be used to immunize animals against infection.

The present invention provides in one aspect, a recombinant virus, characterised in that it includes a coding sequence for a hybrid polypeptide, said hybrid polypeptide comprising at least one immunogenic polypeptide segment which is foreign to the virus or virus infected cells in association with a surface or membrane-associated polypeptide segment to locate said hybrid polypeptide on or at the surface of virus infected cells.

In a particular aspect, this invention provides a recombinant vaccinia virus, characterised in that it includes a coding sequence for a hybrid polypeptide, said hybrid polypeptide comprising at least one immunogenic polypeptide segment which is foreign to vaccinia virus or vaccinia virus infected cells, in association with a surface or membrane-associated polypeptide segment to locate said hybrid polypeptide on or at the surface of vaccinia virus infected cells.

In another aspect, this invention provides a DNA molecule comprising a coding sequence for a hybrid polypeptide, said hybrid polypeptide comprising at least one immunogenic polypeptide segment which is foreign to vaccinia virus or vaccinia virus infected cells in association with a surface or membrane-associated polypeptide segment to locate said hybrid polypeptide on or at the surface of vaccinia virus infected cells.

In yet another aspect, there is provided a hybrid polypeptide comprising at least one immunogenic polypeptide segment which is foreign to vaccinia virus or vaccinia virus infected cells in association with a surface or membrane-associated polypeptide segment to locate said hybrid polypeptide on or at the surface of vaccinia virus infected cells.

The present invention is illustrated by way of example by the expression of a hybrid polypeptide based on the secreted repetitive plasmodial antigen (the S-antigen) in a recombinant vaccinia virus.

The genes for a large number of asexual blood stage antigens of Plasmodium falciparum have been isolated and sequenced in the hope of identifying host protective antigens (Kemp et al, 1983). A number of these antigen genes have been expressed in recombinant vaccinia virus. Effective immunization may in many instances depend on the foreign antigen being expressed correctly on the surface of the virus infected cell. However, surface proteins of relatively complex organisms such as protozoan parasites may not find their way to the surface when the relevant genes are introduced into mammalian cells. For example,

P.falciparum proteins located in the membrane of the host erythrocyte must traverse a rather complex pathway through the membranes of the parasitophorous vacuole before reaching this final destination, and this pathway is not understood.

The S-antigen proteins of *Plasmodium falciparum* are secreted by the parasite into the space which separates the limiting membrane of the dividing parasites and the inner membrane of the red blood cell initially formed during the invagination process accompanying the parasite invasion of the red blood cell and subsequently elaborated during parasite growth. Immunogold electromicroscopy indicates that this space, known as the parasitophorous vacuole, is filled with S-antigen shortly before rupture of the mature schizont. The sequence of genes for two of these S-antigen molecules (Cowman et al, 1984) indicates the presence of a short region at the 5' end of the gene which would code for a hydrophobic signal peptide but no other significant regions of hydrophobicity in the rest of the gene, consistent with this characterization as a secreted protein. These signals are recognised accurately when this protein is expressed in vaccinia virus infected mammalian cells in *in vitro* culture.

A primary aim in the work leading to the present invention was to investigate the use of the live recombinant vaccine virus in the delivery of plasmodial blood-stage antigens to immunize animals and to compare the immune responses to those obtained using peptides generated by recombinant DNA techniques or synthesized chemically. A theoretical advantage of the live viral delivery approach is that it should better stimulate the

cellular arm of the immune system which may be of prime importance in the efficacy of anti-parasitic vaccines.

Antibody responses to the secreted S-antigen expressed by recombinant vaccinia virus in infected rabbits and mice are small. In both cases antibody titres peak early after infection and wane rapidly. These responses are not boosted by challenging with either a second immunization with recombinant virus or with chemically synthesized concatamers of the 11 amino acid repeating polypeptide in aqueous solution. Boosting with S-antigen β-galactosidase fused polypeptide in FCA does not always result in a larger anti S-antigen response than that seen by immunizing naive mice with a primary injection of this fused polypeptide. It has been found, however, that the addition of a transmembrane domain improves the immunogenicity of the vaccinia S-antigen recombinant, thus indicating the importance of presentation on the surface of the virus infected cell.

The results of this work with the S-antigen suggest the general approach by which a foreign, non-surface, immunogenic polypeptide is associated with the surface of vaccinia infected cells as a hybrid molecule. In the present case, the mouse immunoglobulin gene has been used to provide the sequences necessary for the expression of the immunogenic polypeptide on the infected cell surface. However, other surface antigen molecules that can be efficiently expressed on the surface of vaccinia infected cells could equally well be used to provide such sequences. Ideal candidate molecules would include, for example, a vaccinia virus surface protein, or introduced antigens such as HBSAg.

Further characteristics and features of this invention are described in the following Examples and the accompanying drawings which are presented by way of illustration only and are not to be considered as limiting the scope of the present invention in any way.

EXAMPLE 1

Figure 1 illustrates the construction of transfection plasmids containing the deleted S-antigen gene of the FCQ27/PNG (FC27) isolate of P.falciparum.

(a) shows the structure of the genomic copy of the FC27 S-antigen gene with sequences encoding a signal peptide (dark shading) and approximately 100 copies of the 11 amino acid repeating peptide sequence shown beneath the gene.

(b) shows the 4000bp genomic subclone FC27.4.S described by Cowman et al (1984) containing all non-repeat sequences of the S-antigen gene but only 13 copies of the repeat sequence due to spontaneous deletions which occurred during cloning in E.coli. This DNA was cleaved at the AhaIII restriction endonuclease sites 40 base pairs 5' and 35 base pairs 3' to the coding region of the gene. Following the addition of EcoRI linkers this fragment was cloned into the unique EcoRI restriction site of pGS62 (see Experimental Procedures) to yield the plasmid pV8. In this construct, the S-antigen gene is located immediately downstream from the vaccinia virus 7.5K gene promoter and is flanked on both sides by vaccinia virus TK gene sequences as shown in (c). In a separate cloning described in Detail in Figure 4 and Experimental Procedure, a hybrid S-antigen gene containing an immunoglobulin transmembrane

sequence (hatched) and intracellular domain (dotted) was constructed from pV8 to generate the plasmid pVA20 which is shown in part in (d).

Figure 2 is a Western blot analysis of S-antigen produced by BSC1 cells infected with the recombinant vaccinia virus V8 (lanes 2 and 3) compared with that produced by E.coli under the control of the pUC9 β-galactosidase promoter (lane 1). Lanes 2 and 3 show the relative amounts of S-antigen associated with the virus infected cells and the culture medium at 48 hours after infection. The culture medium was centrifuged at 12,000g for 3mins prior to analysis. Filters were probed with a rabbit antisera recognizing only the 11 amino acid repeat portion of the S-antigen.

Figure 3 shows the time course of the synthesis and secretion of S-antigen in vaccinia infected BSC1 cell monolayers. Cells were infected for 1hr at 1pfu/cell. At this time the innoculum was removed and fresh medium was added. A sample of the culture medium was taken at various times after infection and subjected to centrifugation at 12,000g for 3mins. The supernatent from this centrifugation was taken for analysis. The remaining cells and medium were scraped from the dishes and quantitatively transferred to a fresh tube. Following sonication a sample was taken and dissolved in SDS sample buffer for analysis by SDS/PAGE. Equal fractions of each sample were analysed. Filters were probed with a rabbit antisera which specifically recognized the 11 amino acid repeat of the S-antigen.

Figure 4 is a diagramatic representation of the steps used in the subcloning of the mouse membrane IgG

transmembrane sequence into the Sphl site at the 3' end of the FC27 S-antigen gene. A 186bp HaeIII fragment encoding the transmembrane, intracellular domain and a portion of the hinge region was isolated from the γ1 cDNA clone described by Tyler et al, 1982. Sphl linker DNA was added to the ends of this fragment which after Sphl digestion, was cloned into the Sphl site of the S-antigen gene clone pFC27 Aha2 to generate the new clone pA20. The EcoRI fragments from these plasmids containing the S-antigen gene were then cloned into the EcoRI cloning site of the vector pGS62 (see Figure 1) to generate the plasmids pV8 and pVA20 respectively.

The sequence at the junction of the S-antigen gene and the immunoglobulin gene is shown at the bottom and indicates the new amino acid sequence at the junction. The amino acids alanine and proline, indicated with an asterisk, are not present in either of the parental proteins as they are generated by the Sphl linker DNA sequences. Six amino acids of the extracellular domain of the immunoglobulin gene are present in the new hybrid protein.

Figure 5 shows that the S-antigen produced by cells infected with the VA20 recombinant virus is no longer secreted. Samples of infected cells and culture medium were collected as described in Figure 3 48 hours after infection with either V8 or VA20 recombinant virus at 1pfu/cell. The total amount of S-antigen appears to remain the same, however very little is secreted into the medium in the case of VA20 infected cells. Westerns were probed with a rabbit anti-FC27 S-antigen repeat antisera.

Figure 6: BSC-1 cells infected for 48hrs with either the V8 or VA20 recombinant virus were solubilized in 0.05% Triton X114 for 1hr at 4°C. Insoluble material and nucleii were removed by low speed centrifugation. By elevating the temperature to 37°C a cloudy suspension of insoluble Triton X114 micelles separated by centrifugation at 37°C and each fraction was repurified by a further cycle of Triton X114 partitioning. The S-antigen present in the detergent and aqueous phase was determined by Western blot analysis using the rabbit anti-repeat antisera (R210).

Figure 7 shows indirect immunofluorescence of BSG1 cells infected 18hrs earlier with recombinant virus VA20 (A and C) or V8 (B and D). Cells were either fixed prior to staining (A and B) to permeabilize the cells and allow detection of intracellular S-antigen or fixed after staining to detect S-antigen localized on the surface of the infected cells (C and D).

Fixation was with ice cold 95% ethanol:5% glacial acetic acid. Rabbit 210 antisera at 1:500 dilution was used to localise the S-antigen. A FITC-conjugated goat anti-rabbit conjugate was then used as the second antibody prior to mounting in glycerol containing the fluorescent stabilizer DABCO. Cells were photographed under UV illumination and oil immersion. MagX400.

Figure 8 sets out the antibody titres of mice sera assayed at 3 weeks after a single IP immunization with $1x10^7$ PFU of the V8 or VA20 recombinant virus. Ninety-six well microtitre trays were coated with a FC27 S-antigen repeat/β-galactosidase fusion polypeptide preparation at a predetermined optimal concentration of

3μg/ml. Pre-immune sera were serial diluted to determine the dilution at which half maximal absorbance was reached. Thse values are plotted for both the BALB/c.H-2^k and 129/J strains of mice used.

Figure 9 is a plot of the absorbance values obtained in a typical ELISA assay of rabbit antisera taken at one to five weeks after a single ID injection of 10^8 PFU of live recombinant virus VA20. Sera were assay for both anti S-antigen antibodies as described in Figure 8 at a standard dilution of 1:320 of the sera (dotted line) or for anti-vaccinia antibodies using plates coated with BPL inactivated vaccinia virus at a standard dilution of 1:2580 of the serum (solid lines). (B) shows the absorbance values obtained in a ELISA assay in which individual rabbit antisera were assayed for anti S-antigen antibodies at 2 weeks after intradermal immunization with 10^8 PFU of recombinant virus V8 (dotted lines) or VA20 (solid lines).

Experimental Procedures

Plasmid constructions. A 880bp AhaIII fragment containing a deleted version of the FC27 S-antigen gene was isolated from the genomic EcoRI clone FC27.4.S described by Cowman et al. (1984). EcoRI linkers were added prior to cloning this fragment into pUC9. This 880bp subclone (pFC27 Aha2) encoded the complete 3' end of the S-antigen including the 23 amino acid hydrophobic signal sequence and the 68 amino acid conserved amino terminus, 13 copies of the 11 amino acid repeating peptide of which we estimate there are 100 copies in the undeleted protein, and the complete 35 amino acid sequence of the conserved carboxy terminal end of the molecule. As well there were 40 and 35 base pairs of 5' and 3' noncoding flanking DNA respectively. This 880bp EcoRI fragment was then cloned into the single EcoRI cloning site of the vaccinia transfection vector pGS62 which was constructed by deleting one of

the EcoRI restriction enzyme sites in the vector pGS20 described by Mackett et al. (1984).

Recombinants were selected in which the S-antigen gene was inserted in the correct orientation 3' to the vaccinia 7.5K protein early gene promotor and flanked on either side by the 5' and 3' ends of the vaccinia virus TK gene sequences of the plasmid vector. This new construct, pV8, was used to transfect CV1 cells infected with wild type vaccinia virus giving rise to the recombinant vaccinia virus V8, containing the S-antigen gene.

The addition of the mouse membrane immunoglobulin transmembrane sequence to the S-antigen gene. A 186bp HaeIII fragment containg sequences encoding six amino acids of the hinge region, 26 amino acids of the transmembrane domain and 28 amino acids of the intracellular domain of the mouse IgG, immunoglobulin was isolated from the γ1 cDNA clone described by Tyler et al (1982). SphI linker DNA with the sequence 5'-CCGCATGCGG-3' was then ligated to the HaeIII fragment, digested with SphI and cloned into the unique SphI site located 65bp from the 3' end of the S-antigen gene in the subclone pFC27 Aha2.

The resultant clone pA20 containing the inserted Sphl fragment in the correct orientation with respect to the S-antigen gene, was then digested with EcoRI and the ~1080bp fragment was cloned in the correct orientation into the EcoRI site of the pGS62 vector described above to yield the plasmid pVA20. This plasmid DNA was used to transfect vaccinia infected CV1 cells to produce the recombinant vaccinia virus VA20.

Methods for the production and selection of recombinant vaccinia virus. Methods are as described by Mackett et al (1984) with the exception that single virus plaques were selected by two rounds of end point dilution in 96-well microtitre trays containing monolayers of TK^- 143 cells in the presence of 25μg/ml 5-bromodeoxyuridine (BUdR). Recombinant viruses containing the S-antigen genes were screened for the presence of DNA by dot

blot analysis or for the production of S-antigen which was detected by a high titre polyclonal antisera, R210, raised by immunizing rabbits with a β-galactosidase fused polypeptide from clone Ag16 (Coppel et al, 1983) containing 23 copies of the 11 amino acid FC27 S-antigen repeating polypeptide.

Expression of S-antigen in recombinant vaccinia infected cells. Confluent monolayers of BSC-1 cells were routinely infected at 1pfu/cell with purified recombinant virus and allowed to incubate at 37°C for 18-48hrs at which time the infected cells and/or the supernatant were harvested and dissolved in SDS, sample buffer and boiled. Samples were then analysed by immunoblotting and probed with a rabbit anti S-antigen antisera, R210 which recognizes the repeating epitope of the S-antigen molecule.

Triton X114 partitioning. Recombinant vaccinia infected cells were dissolved in 0.5% Triton X114 in PBS for 1hr at 4°C. Following centrifugation at 2000 rpm to remove nuclei in the Triton X114 soluble material was layered over a cushion of 6% Triton in 0.06% sucrose/PBS and then the temperature was raised to 37°C. The cloudy suspension of insoluble material was removed by centrifugation at 37°C. This fraction which is referred to as the Triton X114 pellet, should contain the integral membrane proteins by virtue of the greater affinity of their hydrophobic transmembrane sequences for the Triton X114 detergent which becomes insoluble at elevated temperature (Bordier, 1981). The supernatant which should contain soluble proteins was also collected. Each fraction was subjected to a further cycle of purification to reduce contamination. Samples of each fraction were then added to SDS sample buffer and analysed by immunoblotting.

Immunofluorescence. BSC-1 cells were grown onto sterile glass coverslips for a period of 6hrs after which they were infected at 0.5pfu/cell with either the V8 or VA20 recombinant viruses or TK^- nonrecombinant virus as control. After 18hrs, coverslips were rinsed in cold PBS and then stained immediately with rabbit anti-S-antigen antisera followed by FITC conjugated

sheep anti-rabbit antibodies. Cells were then post fixed in cold 95% ethanol:5% glacial acetic acid prior to mounting under glycerol and visualization by fluorescence microscopy.

A parallel group of infected cells was fixed in cold 95% ethanol:5% glacial acetic acid prior to staining to permeabilize the cells.

Immunization of animals

Rabbits were immunized with a single intradermal injection of 10^8 PFU of purified recombinant or TK^- wild type virus on their lower back followed by a second immunization six weeks later. Lesions appeared beneath the skin within a few days of the first immunization, reaching a size of approximately 1 to 1.5cm in diameter. Occasionally these lesions ulcerated. Lesions were no longer apparent after two weeks. Rabbits were bled at weekly intervals and the sera analysed for anti-S-antigen or anti-vaccinia antibodies in an ELISA assay. Age and weight-matched inbred mice of various strains were immunized by a single IP injection of $1x10^7$ PFU of virus followed by a second challenge three weeks later. Sera were usually collected three weeks after the primary immunization and 12 days and three weeks after the rechallenge. Anti-S-antigen and anti-vaccinia antibody titres were assayed by serial dilution of the sera in an ELISA assay.

Results

The FC27 S-antigen gene of *P.falciparum* is expressed in recombinant vaccinia virus.

A 880bp AhaIII fragment of the FC27 genomic clone FC27.4.S (Cowman et al, 1985) (Figure 1b) was cloned into the EcoRI site of the vaccinia virus transfection plasmid pGS62 (a derivative of pGS20 described by Mackett et al, 1984). In this construct the initiation codon for the S-antigen gene lies 40bp downstream from the EcoRI cloning site which is adjacent to the vaccinia 7.5K gene promotor (Figure 1c). Initiation of translation at this methionine codon and termination at the termination codon 822 nucleotides downstream should result in a protein of 274 amino acids in length or approximately 28K dalton in size following cleavage of the signal peptide. This protein should contain 13 copies of the 11 amino acid repeating polypeptide. A cDNA

clone designated Ag16 that encodes this repeating epitope has been described previously (Coppel et al, 1983). From the sequence of this cDNA, the P.falciparum segment of its stable β-galactosidase fused polypeptide must consist entirely of repeating 11 amino acid polypeptides. Rabbit antibodies to this fused polypeptide recognise the 220K dalton native S-antigen molecule (Coppel et al, 1983). These antibodies reacted specifically with proteins produced by the recombinant vaccinia virus V8 in both plaque immunoassays and in immunoblots of proteins isolated from V8 infected cells (Figure 2b, lane 2). The apparent size of the molecule is however much greater than the 27K dalton predicted from the sequence. Moreover, a number of less abundant smaller and larger bands were also present. The aberrant molecular weight is not due to glycosylation as proteins of the same apparent molecular weight are made in Escherichia coli under the control of β-galactosidase promotor elements of pUC9 (Figure 2, lane 1). Moreover, the DNA insert in the recombinant virus was indeed the correct length (data not shown). We assume that abnormal SDS binding characteristics result in this aberrant MW determination on SDS/PAGE. Thus the S-antigen appears to be synthesized in recombinant vaccinia infected cells under the control of vaccinia promotor elements.

The S-antigen is secreted from vaccinia infected cells

Monolayers of BSC1 cells were infected with purified recombinant virus V8. After 1hr, the virus innoculum was replaced with fresh medium and then at various times the cells and culture medium were harvested, separated by centrifugation and subjected to analysis by immunoblotting. Detectable amounts of S-antigen began to appear in the medium at 3-4hrs after infection (Figure 3) and increased over the next 48hrs to reach a total of over 65% of the total S-antigen synthesized (Figure 2b and c). Control experiments with anti-vaccinia antibodies showed that this was not due to virus present in the supernatant material (data not shown).

Clearly the S-antigen is secreted from the vaccinia infected eukaryotic cell in much the same way as it is from the parasite into the parasitophorous vacuole late in schizogeny. This data indicates that the recognition signals such as the signal polypeptide are recognized despite the species differences.

Immunization of animals with the V8 recombinant virus

Three rabbits were immunized as described in experimental procedures. The antibody titres were not above preimmune levels in two out of three cases and less than 1:50 in a third rabbit. Anti-vaccinia antibodies reached a very high level in all three animals. Thirteen strains of mice with 3 animals in each group were also vaccinated. Only marginal increases in antibody titre above preimmune values were seen at a 1:20 dilution of serum.

We concluded that despite the high level of expression of the S-antigen, this secreted molecule was not recognized efficiently by the immune system presumably because it was not presented properly on the surface of the virus infected cells.

The addition of a transmembrane sequence to the S-antigen

A fragment of a mouse γ1 cDNA clone containing sequences encoding part of the hinge region and the whole of the transmembrane and intracellular domains, was cloned in frame into the SphI site at the 3' end of the S-antigen gene with the aid of SphI blunt end adaptors to generate the hybrid gene, pVA20 (see Figure 4). This gene was then introduced into the same cloning site in the pGS62 vector as the V8 clone and transfected into vaccinia infected CV1 cells as described in Experimental Procedures. The level of expression of this hybrid protein was similar to that of the V8 recombinant, however the protein was no longer secreted from the vaccinia infected cells (Figure 5).

Triton X114 partition experiments (Bordier, 1981) were performed to test if, by this criteria, the hybrid S-antigen containing the transmembrane segment behaved as a typical integral membrane protein. Indeed, whereas

the V8 protein behaves exclusively as a hydrophilic soluble protein, the majority of the VA20 protein partitioned into the detergent phase (Figure 6) indicating that the hydrophobic transmembrane sequence had converted the soluble S-antigen protein into a membrane-associated protein. BSC-1 cells infected with either VA20 or V8 recombinant virus were subjected to indirect immunofluroescence 18hrs after infection. Cells were either fixed prior to staining in cold 95% ethanol-5% glacial acetic acid to permeabilize the membranes and allow cytoplasmic labelling with antibodies or fixed after staining to reveal only surface bound antigen. The results, shown in Figure 7, indicate that there was no surface labelling of the V8 infected cells, and obvious labelling on the surface of VA20 infected cells. At higher antibody concentrations a small but significant level of surface labelling could be seen on V8 infected cells.

Immunization of animals with VA20

Two strains of mice which gave differential responses to vaccinia antibodies after the primary immunization with V8 recombinant virus were chosen to analyse the immunogenicity of the VA20 virus. The response of these mice to the IP injection of 10^7 PFU of the V8 and VA20 virus is shown in Figure 8. Three rabbits were also challenged and the results are shown in Figure 9. Figure 9a shows that the anti S-antigen Ab titres in the VA20 immunized rabbits peaked two weeks after immunization despite the fact that anti-vaccinia antibody titres continued to climb over the next three week period. The same was true of the rabbit responses to the V8 recombinant although here the responses were very small and difficult to measure. In Figure 9B, the sera giving maximum responses in all six rabbits receiving the V8 and VA20 recombinants are compared in an ELISA assay by serially diluting the serum. Despite large differences between the individual titres of sera from the three rabbits, a clear increase in the immunogenicity of the protein is apparent.

EXAMPLE 2

Example 1 is a specific example of a more general method by which biologically important molecules which, although themselves not surface antigen molecules, can be redirected to the surface of recombinant vaccinia virus-infected cells. This example also shows how crucial this surface localization of the antigen is for the induction of good immune responses to the foreign introduced antigen.

The antigen chosen for Example 1, the malarial S-antigen, has to date not been implicated as a potential vaccine candidate primarily because the immunodominant repeat portion of the molecule is remarkably variant. These repeating structures vary enormously in their number, length and amino acid composition which greatly affects their immunological properties but not, it would seem, their behaviour as secreted proteins.

The present Example illustrates that it is possible to replace the S-antigen repeating epitope with an unrelated sequence which is of importance as a vaccine molecule. These hybrid molecules, containing in addition an appropriate trans-membrane anchoring sequence, should be, in many cases, as efficiently transported to the surface of the recombinant virus-infected cell as is the hybrid S-antigen molecule described above.

Set out below are the procedures necessary to delete the repetitive portion of the S-antigen molecule and to replace it with another repetitive epitope. By way of example, the Asn-Ala-Asn-Pro (NANP) sequence of the circumsporozoite coat protein (which others have shown to be the critical epitope in the development of immunity to the infective sporozoite stage of falciparum

malaria), has been chosen. However this new epitope could just as well be derived from molecules of the other malaria life cycle stages or from antigens of other clinically important pathogens. Thus, this Example is one example of an approach by which one can tailor an antigenic determinant into a "carrier" molecule designed to deliver this epitope to the surface of recombinant vaccinia virus infected cells. We have also shown that another recombinant virus, a murine retrovirus, is also able to express the product of the hybrid VA20 gene on the surface of virus-infected mouse cells in culture demonstrating that this approach may be of general applicability to a variety of antigenic epitopes expressed in any of a number of "carrier" epitopes in a variety of recombinant viral vector systems.

Figure 10 is a diagramatic representation of the manipulations required to delete the 33bp S-antigen repeating sequences from pVA20 and to replace them with sequences encoding 16, 32 and 48 copies of the 4 amino acid repeating epitope of the P.falciparum circumsporozoite coat protein.

Figure 11 is a schematic representation of the P.falciparum circumsporozoite coat protein gene (at top) showing the sequence of the dominant 4 amino acid repeating unit, NANP. Below are shown the sequences of the synthetic oligonucleotides used in the synthesis of the new insert and (at bottom) the sequence of the 5' and 3' junction regions between the BamH1 cut plasmid pLK8 (S-antigen sequences) and the new insert sequences. Note the 6BP adaptor sequences shown in solid boxes at the 5' and 3' ends of the insert, the Sau3A sites flanking the insert and how a BamH1 site is only

regenerated at the 5' end of the insert. 5' and 3' refer to the ends of the coding strand of the insert DNA.

Figure 12 shows double stranded DNA sequencing reactions of DNA from plasmids p6.44, p66.6 and p.666.34 containing 16, 32 and 48 copies, respectively, of the 12bp repeating sequence of the *P.falciparum* circumsporozoite protein. For clarity only the "A" reactions are shown for the 3 constructs. At left is the sequence of the coding strand of the synthetic oligonucleotide used in the constructions with the sequence 5'-AACVCCAACCC-3'. As can be seen, two pairs of A doublets occur in each copy of the repeat. The sequencing primer was a 17 nucleotide homologous to coding strand sequences located 20bp 5' to the BamH1 site.

Figure 13 is an immunoblot of proteins derived from recombinant virus-infected BSC-1 cells probed with an antisera (R516 anti $NANP_3$-KLH) produced by immunizing rabbits with a 12 amino acid long synthetic peptide encoding 3 copies of the 4 amino acid squence NANP conjugated to KLH. This antiserum recognises polypeptides produced by cells infected with recombinant virus (V6.44) containing the -6.44 hybrid gene described above but not produced in a parallel experiment with cells infected with a similar construct containing unrelated sequences inserted at the BamH1 site (V-control). Molecular weights in Kdaltons are shown at right.

Deletion of the S-antigen repeating sequence

Prior to deleting the S-antigen repeat sequence from the pVA20 construct described above, a number of

changes were made to the pGS62 vector used in its construction. First the Cla1 site in the pBR322 portion of the plasmid was removed by partial digestion with Cla1, Klenow "filling-in" and religation. Into the now unique Cla1 site in the vaccinia TK gene portion of the vector a 251bp Taq1 - EcoR1 fragment from the strong hybrid bacterial promoter LacUV5/Trp was inserted, using a synthetic oligonucleotide adaptor (AATTATCGAT) to convert the EcoR1 site to a Cla 1 site (Amann et al 1983). This generated the new vector pGS62 tac (shown at the top of figure 10). Using this vector the expression of genes inserted into the multiple cloning site (MCS) of the vector can be checked in bacteria as well as in virus-infected cells. The BamH1 site was then deleted from the MCS by BamH1 digestion, Klenow "filling-in" and religation generating the new plasmid pGS62-tac[BamH1$_{\Delta}$]. The 1088 bp EcoR1 fragment from pVA20 was cloned into the EcoR1 site of the MCS to generate the plasmid pCL4.

In a parallel cloning experiment the isolated EcoR1 fragment of pVA20 was digested with Sau3A. This enzyme cuts once in each of the S-antigen repeats. The non-repeat fragments 5' and 3' to the repeat portions were isolated and ligated into the EcoR1 site of the vector pGS62-tac[BamH1$_{\Delta}$] to generate the new construct pLK8 shown in figure 10.

The plasmid pLK8 contains the S-antigen gene with less than one full copy of the 33bp repeating sequence and with a unique BamH1 site located within this remaining partial repeat sequence. It is into this site that appropriately engineered sequences encoding antigenic determinants can be cloned, thus neatly replacing the S-antigen repeating epitope.

The incoming sequences in the situation described in this example would need to have BamH1 "sticky ends" and to be engineered in such a way that the reading frame of the whole hybrid gene is maintained. This requires that the total length of the insert DNA should be an equal multiple of 3 base pairs and that the reading frame be in phase with the GAT codon of the GGATCC BamH1 site at the 5' end of the (coding strand of the) insert.

It is also believed that the epitope to be expressed should be repeated as many times as possible to maximize its immunogenicity even if this epitope is represented only once in the native antigen molecule.

By way of example, a 12bp sequence encoding the dominant NANP amino acid repeated epitope of the P.falciparum circumsporozoite coat protein has been chosen. It will be appreciated, however, that the same procedures could equally well be applied to the linear epitopes or the "mimotopes" of conformational epitopes of other antigen molecules.

In this example we have chosen to chemically synthesize the short 12bp sequence encoding the NANP peptide which enables us to "mammalianize" the codons for optimal expression in recombinant vaccinia virus-infected mammalian cells. This may be of importance in the optimal expression of foreign proteins from species such as P.falciparum which exhibit a strong bias in their preferred codon usage away from that seen in mammalian cells. However, the insert could also be derived from naturally occurring sequences as long as it fulfils the length and phase requirements described above.

Synthesis of a new repeating epitope and its insertion into the body of the S-antigen gene

The following procedures are described diagramatically in figure 11.

Two complimentary oligonucleotide sequences 5'-AACGCCAACCCC-3' and 5'-GGTTGGCGTTGG-3' were made on an Applied Biosystems oligonucleotide synthesiser and purified by HPLC. These were then kinased prior to annealing and ligation in the standard way. The oligonucleotides were designed so that the ends were complimentary in one orientation only, ensuring that only "head to tail" ligation of the double stranded monomers was possible. The ligated fragments were then size fractionated on a low gelling temperature agarose gel and DNA molcules in the size range from 180 to 600bp were isolated and purified from the agarose. These size fractionated molecules were then ligated with BamH1 cut/calf intestinal phosphatase treated pLK8 plasmid DNA in the presence of two kinased synthetic oligonucleotides with the sequence 5'-GATCCC-3' and 5'-GATCGG-3'. These adaptors were designed to allow the ligation of the 3' overhanging CC and GG ends of the repeating oligonucleotide fragment to ligate to the 5' overhanging GATC sticky ends of pLK8 and to ensure that the insert sequence was "in frame" with the S-antigen sequence.

Recombinant bacterial clones containing the 12bp sequence were selected by colony hybridization using a gamma-[^{32}P]-ATP kinased oligonucleotide with the sequence 5'-AACGCCAACCCC-3'.

Plasmid DNA was isolated from the positive clones and digested with restriction enzymes to determine the clones which contained the longest inserts. A number of these were then sequenced using the double stranded DNA

sequencing procedure on alkaline denatured plasmid DNA preparations to select a clone with an insert in the correct orientation and to confirm its predicted sequence. This plasmid DNA (p6.44, figure 10) was then transfected directly into wild type vaccinia virus-infected cells to product TK^- recombinant virus as described above.

Increasing the length of the repeating epitope by further subcloning

The new 192bp insert in the hybrid gene p6.44 is flanked by Sau3A sites which allow the insert to be isolated and purified from the recombinant plasmid. However only one BamH1 site at the 5' of the coding strand of the insert is regenerated in this hybrid (see figure 11). Thus the recombinant plasmid can be linearised with BamH1, phosphatase treated and ligated with the isolated 192bp Sau3A fragment. Plasmid DNA was prepared from the transformed bacteria resulting from this cloning and digested with restriction enzymes to select clones with double (or triple) inserts. These were then sequenced to determine which were in the correct orientation and to confirm the predicted sequence. These new hybrids again have a unique BamH1 site at the 5' end of the insert and this process can be repeated many times over, increasing the size of the insert to any desired length. In this example inserts containng 16(p6.44), 32(p66.6) and 48(p666.34) copies of the 12bp repeat of the CSP gene have been poduced (see figures 10 and 12).

Characterisation of the hybrid antigen and its expression in recombinant virus-infected cells.

Once introduced in recombinant vaccinia viruses these hybrid genes are tested in virus-infected cells to see if a stable hybrid protein capable of being recognised by antibodies which are specific for the epitope is produced. An example of this is described in figure 13 which shows a Western blot of proteins produced by V6.44 virus-infected mammalian BSC-1 cells probed with a rabbit antisera raised against a 12 amino acid long synthetic peptide comprising 3 copies of the NANP peptide.

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CLAIMS:

1. A recombinant virus, characterised in that it includes a coding sequence for a hybrid polypeptide, said hybrid polypeptide comprising at least one immunogenic polypeptide segment which is foreign to the virus or virus infected cells in association with a surface or membrane-associated polypeptide segment to locate said hybrid polypeptide on or at the surface of virus infected cells.

2. A recombinant vaccinia virus, characterised in that it includes a coding sequence for a hybrid polypeptide, said hybrid polypeptide comprising at least one immunogenic polypeptide segment which is foreign to vaccinia virus or vaccinia virus infected cells, in association with a surface or membrane-associated polypeptide segment to locate said hybrid polypeptide on or at the surface of vaccinia virus infected cells.

3. A recombinant virus according to claim 1 or claim 2, wherein said hybrid polypeptide coding sequence includes a coding sequence for at least one immunogenic polypeptide of P.falciparum.

4. A recombinant virus according to claim 3, wherein said coding sequence for at least one immunogenic polypeptide of P.falciparum is a sequence which codes for at least one copy of a repeat portion of an immunogenic polypeptide of P.falciparum.

5. A recombinant virus according to claim 4, wherein said coding sequence for at least one immunogenic polypeptide of P.falciparum is a sequence

which codes for more than one copy of said repeat portion.

6. A recombinant virus according to claim 3, wherein said immunogenic polypeptide of P.falciparum is an asexual blood stage antigen of P.falciparum.

7. A recombinant virus according to claim 3, wherein said immunogenic polypeptide of P.falciparum is the circumsporozoite coat protein of P.falciparum.

8. A recombinant virus according to claim 1 or claim 2, wherein said hybrid polypeptide coding sequence includes a transmembrane coding sequence.

9. A recombinant virus according to claim 1 or claim 2, wherein said hybrid polypeptide coding sequence includes a sequence coding for a surface or membrane-associated polypeptide selected from mouse immunoglobulin, vaccinia virus surface protein and hepatitis B surface antigen.

10. A DNA molecule comprising a coding sequence for a hybrid polypeptide, said hybrid polypeptide comprising at least one immunogenic polypeptide segment which is foreign to vaccinia virus or vaccinia virus infected cells in association with a surface or membrane-associated polypeptide segment to locate said hybrid polypeptide on or at the surface of vaccinia virus infected cells.

11. A hybrid polypeptide comprising at least one immunogenic polypeptide segment which is foreign to vaccinia virus or vaccinia virus infected cells in

association with a surface or membrane-associated polypeptide segment to locate said hybrid polypeptide on or at the surface of vaccinia virus infected cells.

1/11

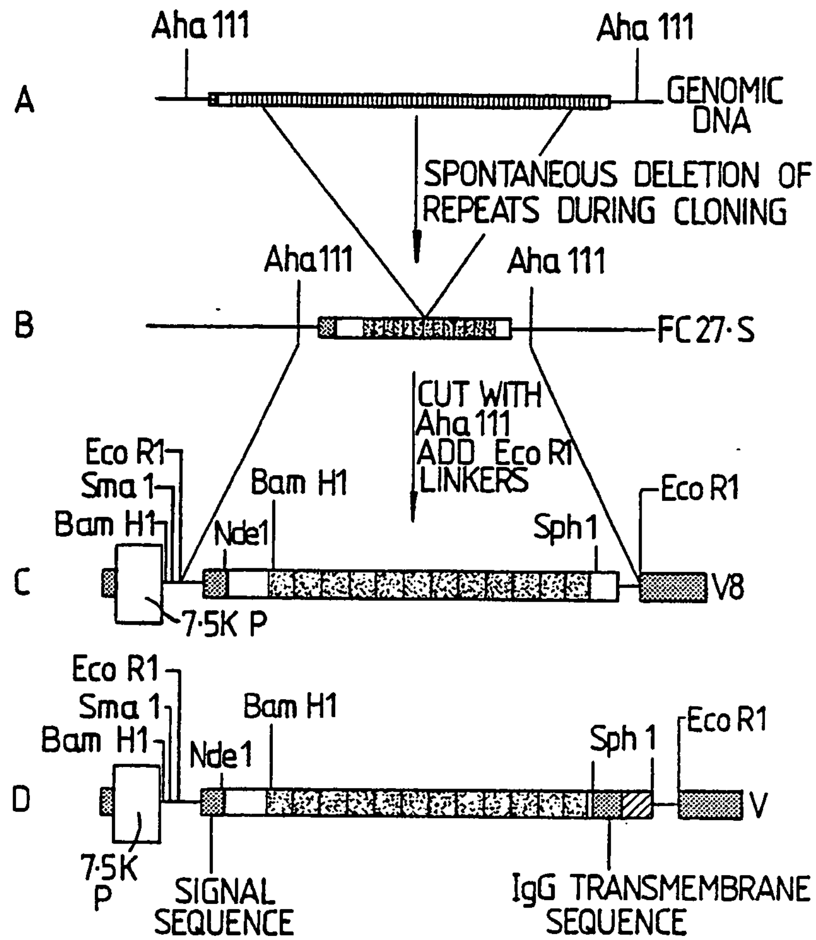

Aha 111
Aha 111
A
GENOMIC DNA
SPONTANEOUS DELETION OF REPEATS DURING CLONING
Aha111
Aha 111
B
FC27·S
CUT WITH Aha 111 ADD Eco R1 LINKERS
Eco R1
Sma 1
Bam H1
Bam H1
Nde1
Sph 1
Eco R1
C
7·5K P
V8
Eco R1
Sma 1
Bam H1
Bam H1
Nde1
Sph 1
Eco R1
D
V
7·5K P
SIGNAL SEQUENCE
IgG TRANSMEMBRANE SEQUENCE

FIG. 1.

2/11

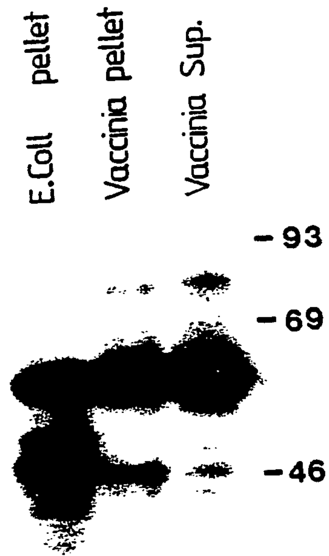


FIG. 2.

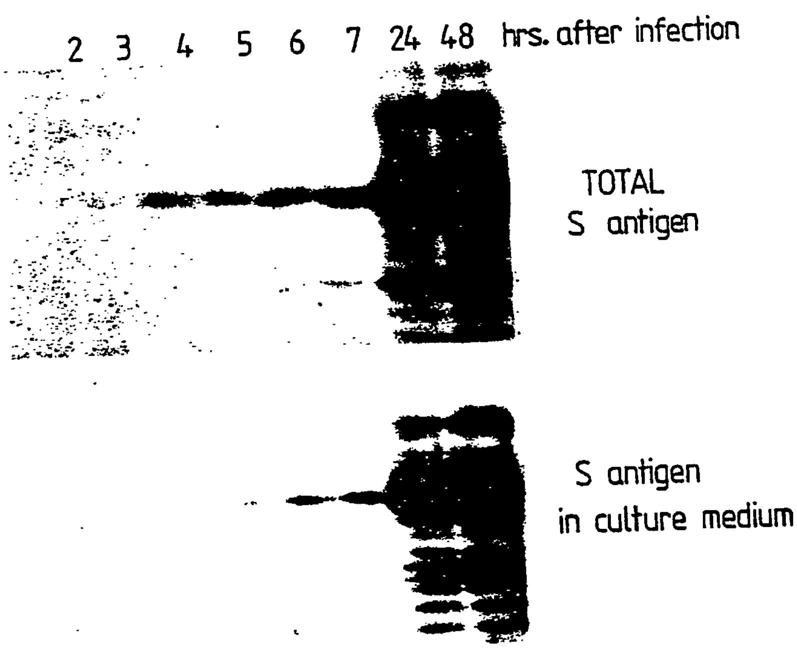


FIG. 3.

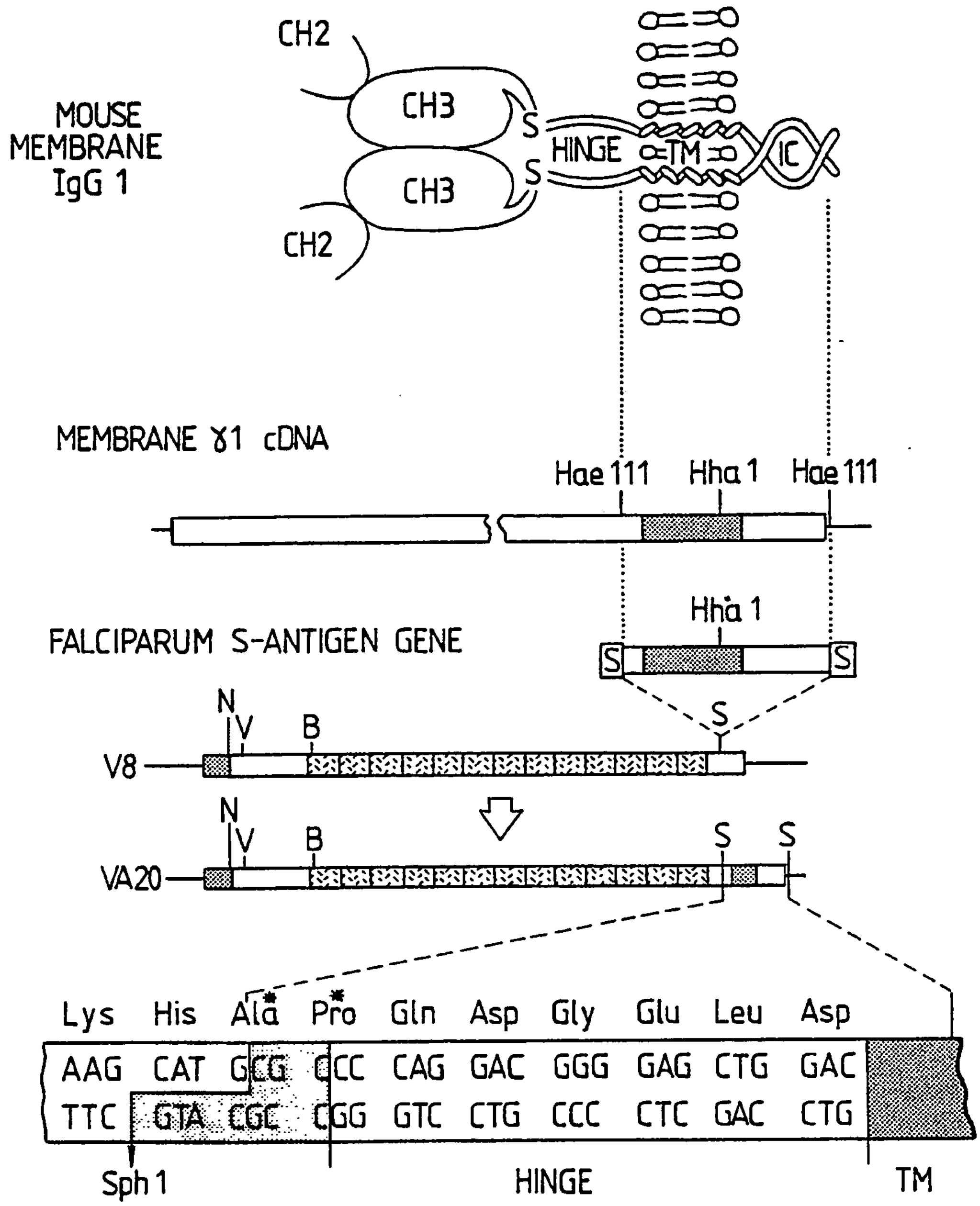

MOUSE MEMBRANE IgG 1
CH2
CH3
S
HINGE
TM
IC
CH3
S
CH2
MEMBRANE γ1 cDNA
Hae 111
Hha 1
Hae 111
FALCIPARUM S-ANTIGEN GENE
Hha 1
S
S
N
V
B
S
V8
N
V
B
S
S
VA20
Lys His Ala* Pro* Gln Asp Gly Glu Leu Asp
AAG CAT GCG CCC CAG GAC GGG GAG CTG GAC
TTC GTA CGC GGG GTC CTG CCC CTC GAC CTG
Sph 1
HINGE
TM

FIG. 4.


cells m. cells m.
-92·5
-69·0
-43·0
secretion

FIG. 5.

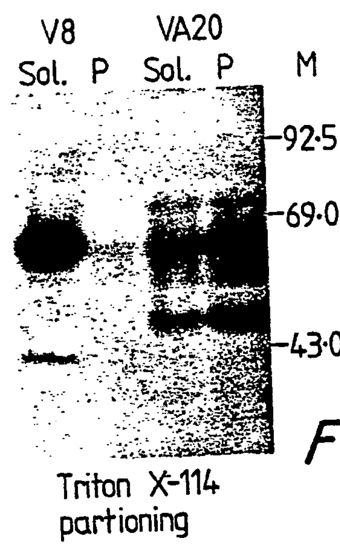

V8
VA20
Sol. P Sol. P M
-92·5
-69·0
-43·0
Triton X-114
partioning

FIG. 6.

A

C

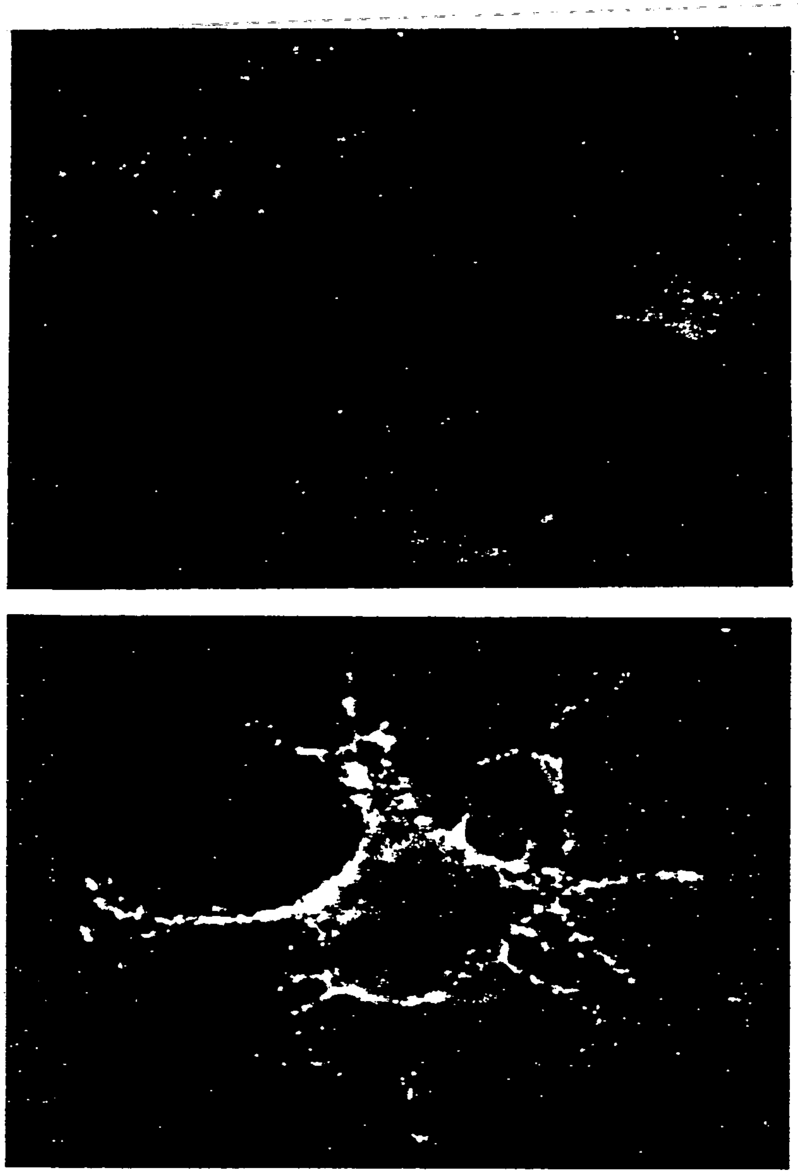
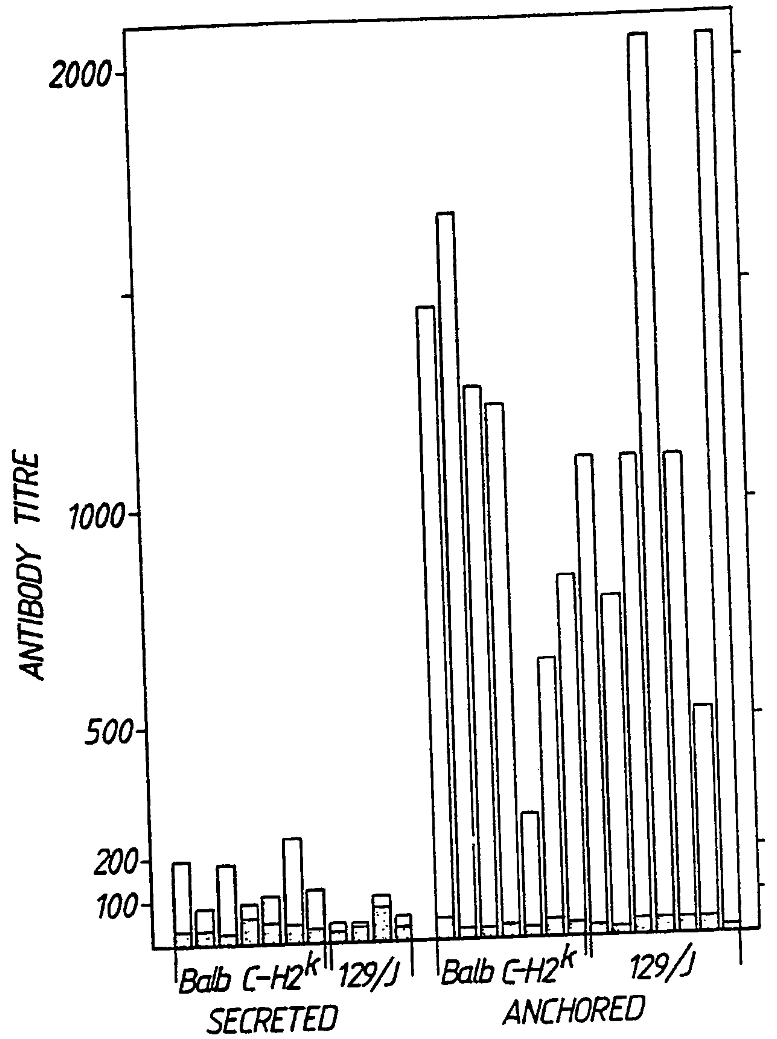


FIG. 7.



2000
1000
500
200
100
ANTIBODY TITRE
Balb C-H2^k
129/J
SECRETED
Balb C-H2^k
129/J
ANCHORED

FIG. 8.

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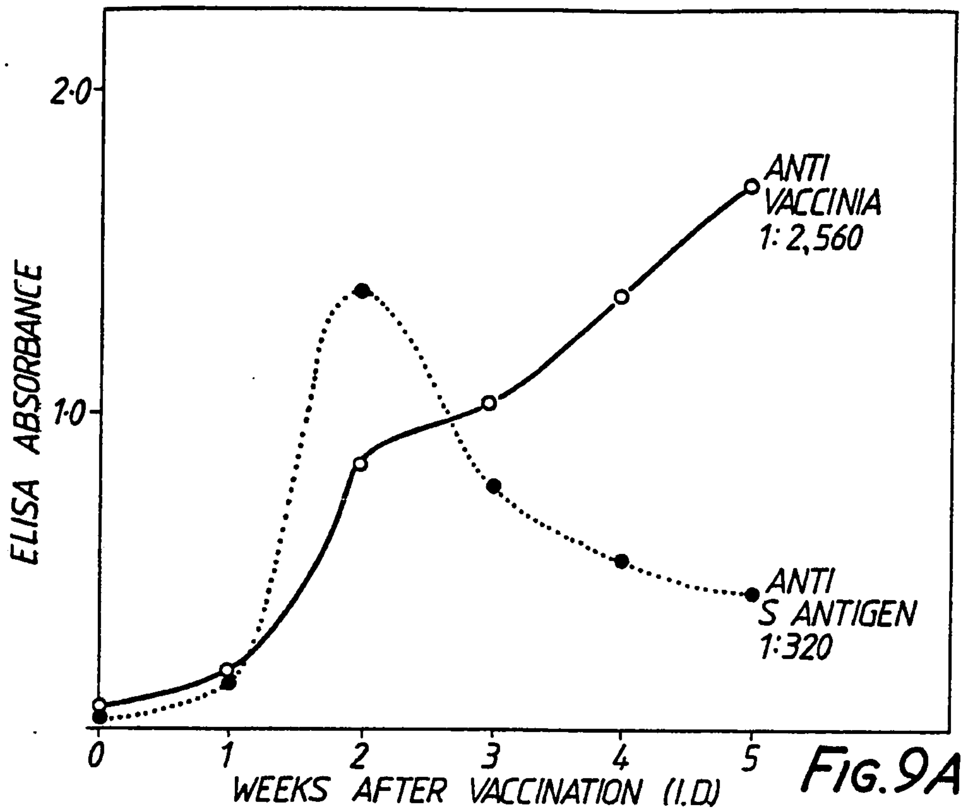


FIG. 9A.

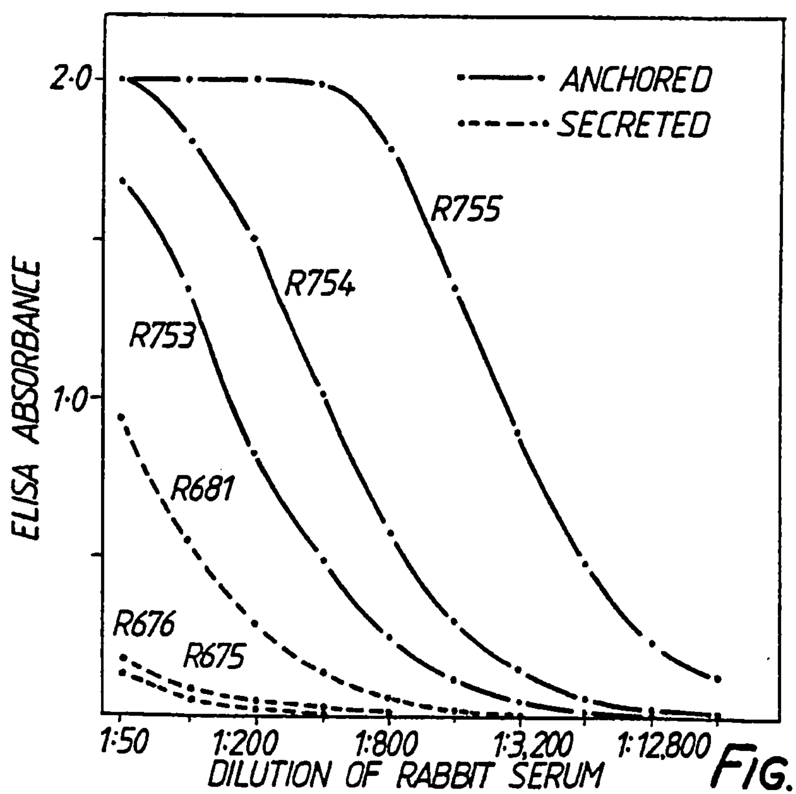
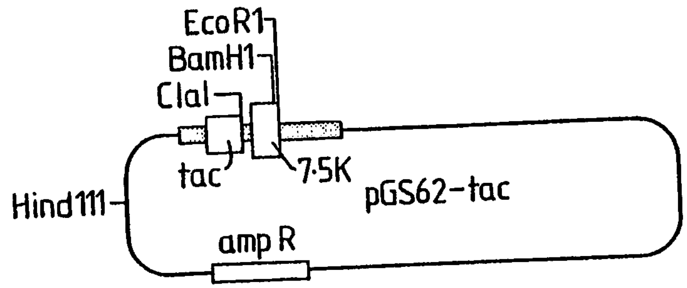
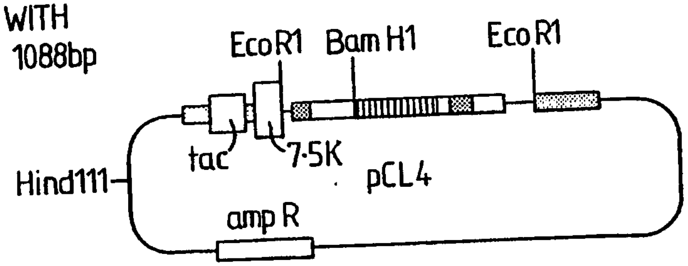


FIG. 9B.



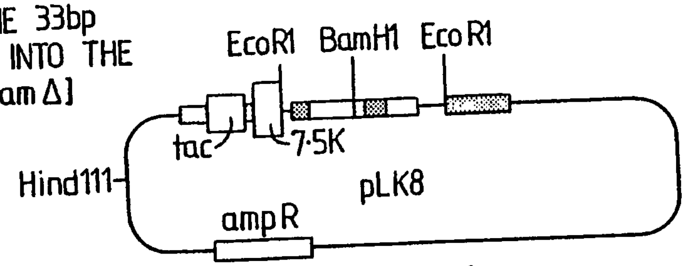
MODIFIED pGS60 VACCINIA VIRUS TRANSFECTION VECTOR (MACKETT ET AL. 1984. J.VIROL 49, 857

DELETE THE BanH1 SITE OF pGS62-tac BY KLENOW TREATMENT, CUT THE NEW PLASMID (pGS62-tac[BamH1Δ] WITH Eco R1 AND LIGATE WITH THE 1088bp Eco R1 INSERT OF pVA20.



S-ANTIGEN GENE IN pGS62-tac [BamH1Δ] WITH A UNIQUE BamH1 SITE IN THE 5' COPY OF THE 13 S-ANTIGEN REPEATS

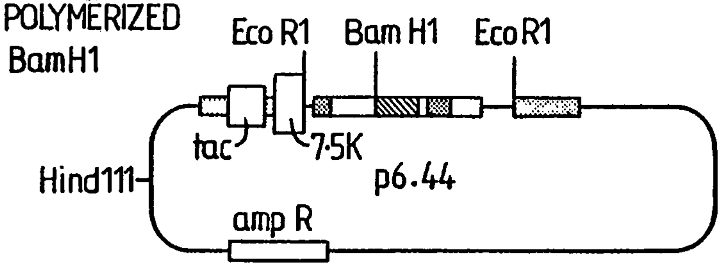
ISOLATE THE 1088bp EcoR1 FRAGMENT. DIGEST WITH Sau3A (WHICH CUTS IN EACH OF THE 13 COPIES OF THE 33bp S-ANTIGEN REPEAT), RELIGATE INTO THE EcoR1 SITE OF pGS62-tac [Bam Δ]



pCL4 WITH 12 OF THE 13 COPIES OF THE 11aa S-ANTIGEN REPEAT DELETED BUT STILL WITH A UNIQUE BamH1 SITE

FIG. 10a.

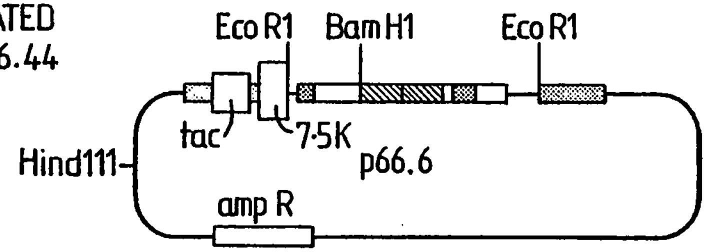
BamH1 DIGESTION AND CIP TREATMENT FOLLOWED BY LIGATION WITH POLYMERIZED 12bp SEQUENCES> 180bp AND BamH1 ADAPTOR SEQUENCES.



ANCHORED S-ANTIGEN GENE CONTAINING 16 COPIES OF A 12bp SEQUENCE ENCODING THE NANP REPEAT OF THE CIRCUMSPOROZOITE COAT PROTEIN

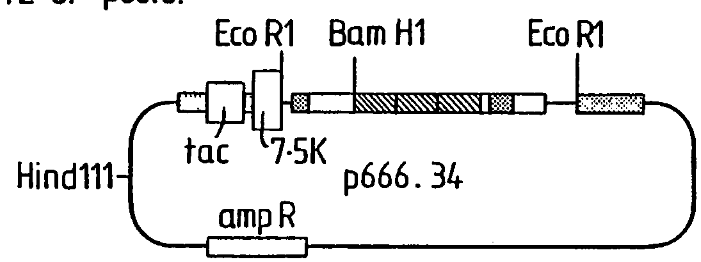
ISOLATED THE 192bp Sau 3A FRAGMENT

CLONE INTO THE CIP TREATED UNIQUE BamH1 SITE OF p6.44



ANCHORED S-ANTIGEN GENE CONTAINING 32 COPIES OF A 12bp SEQUENCE ENCODING THE NANP REPEAT OF THE CIRCUMSPOROZOITE COAT PROTEIN

CLONE INTO THE CIP TREATED UNIQUE BamH1 SITE OF p66.6.



ANCHORED S-ANTIGEN GENE CONTAINING 48 COPIES OF A 12 bp SEQUENCE ENCODING THE NANP REPEAT OF THE CIRCUMSPOROZOITE COAT PROTEIN

FIG. 10b.

10/11

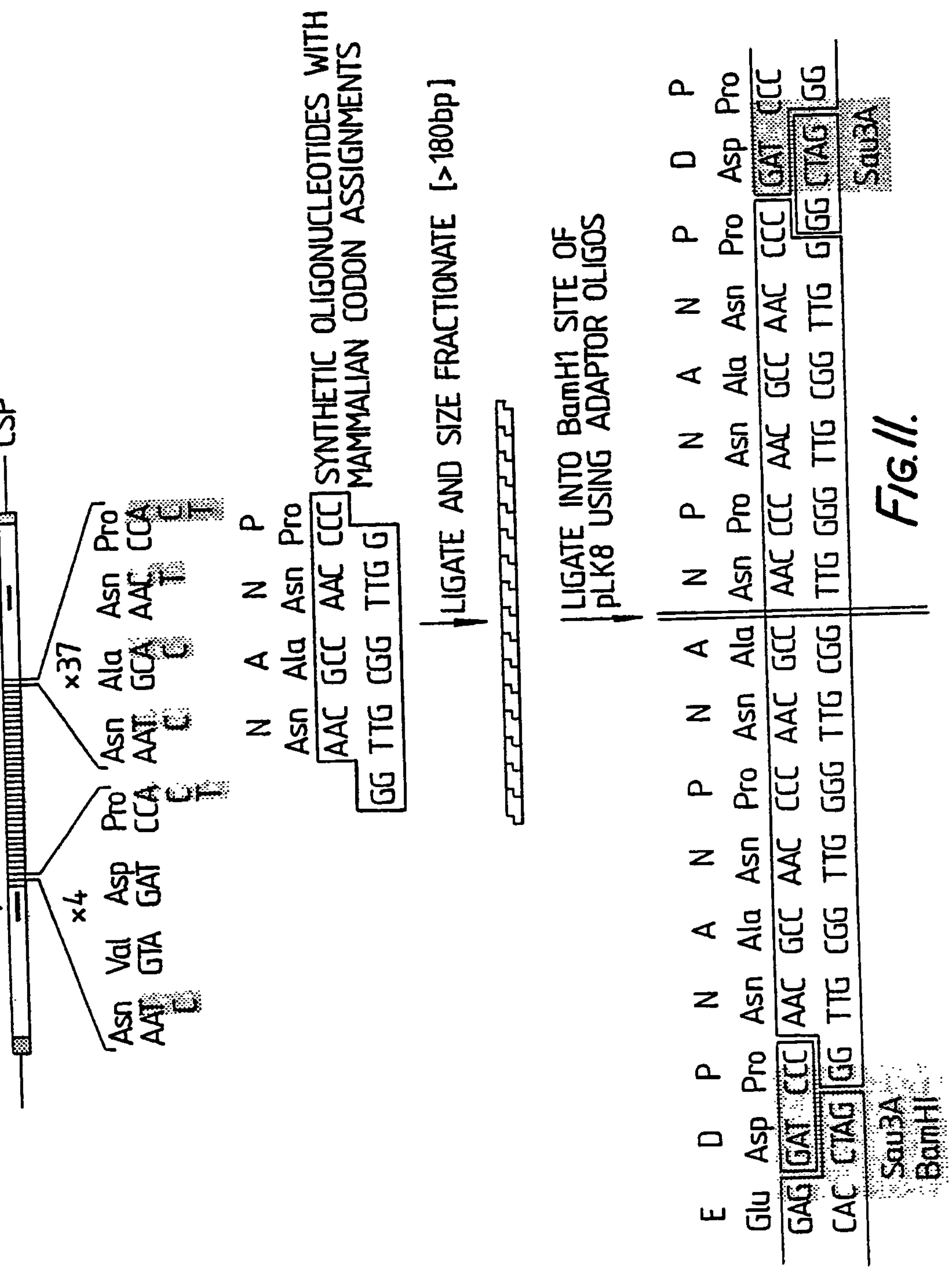
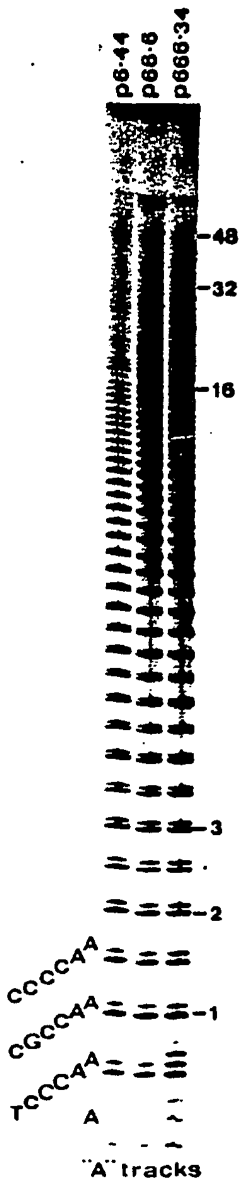
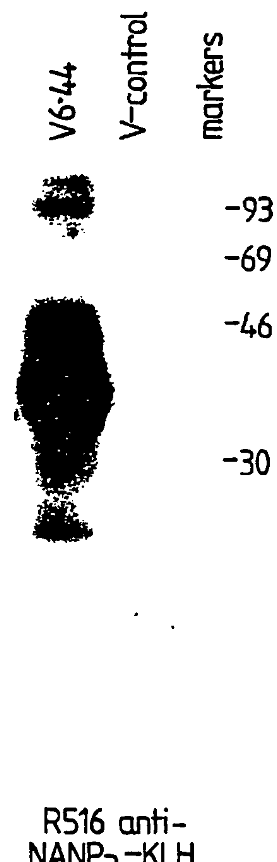

1
2
CSP
×4
×37
Asn Val Asp Pro Asn Ala Asn Pro
AAT GTA GAT CCA AAT GCA AAC CCA
C C C C T C
T T
N A N P
Asn Ala Asn Pro
AAC GCC AAC CCC
GG TTG CGG TTG G
SYNTHETIC OLIGONUCLEOTIDES WITH MAMMALIAN CODON ASSIGNMENTS
LIGATE AND SIZE FRACTIONATE [>180bp]
LIGATE INTO BamH1 SITE OF pLK8 USING ADAPTOR OLIGOS
E D P N A N P N A N P N A N P D P
Glu Asp Pro Asn Ala Asn Pro Asn Ala Asn Pro Asn Ala Asn Pro Asp Pro
GAG GAT CCC AAC GCC AAC CCC AAC GCC AAC CCC AAC GCC AAC CCC GAT CCC
CAC CTAG GG TTG CGG TTG GGG TTG CGG TTG GGG TTG CGG TTG G GG CTAG GG
Sau3A
BamHI
Sau3A

FIG. 11.



p6·44
p66·6
p666·34
-48
-32
-16
-3
-2
-1
CCCCAA
CGCCAA
TCCCAA
A
"A" tracks

FIG.12.



V6·44
V–control
markers
-93
-69
-46
-30
R516 anti-
NANP3-KLH

FIG.13.

INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 86/00256

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [8]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[4] C12N 7/00, 5/00, 15/00, C12P 21/00, 19/34, C07H 21/04, C07K 15/12, A61K 39/015, 39/285 // C12R 1/91

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC	WPI, WPIL Database Keywords Vaccinia or Pox( ) Virus

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

AU: IPC C12N 15/00

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No [13]
X,Y	AU,A, 11596/83 (ANIMAL VACCINE RESEARCH CORPORATION) 21 July 1983 (21.07.83)	(1,10,11)
X,Y	EP,A, 12078 (INSTITUT PASTEUR) 11 June 1980 (11.06.80)	(1,10,11)
Y,P	US,A, 4603112 (HEALTH RESEARCH INCORPORATED) 29 July 1986 (29.07.86)	(1,2,10,11)
Y,P	AU,A, 42335/85 (TRANSGENE S.A.) 7 November 1985 (07.11.85)	(1,2,10,11)
Y,P	EP,A, 162738 MOLECULAR GENETICS RESEARCH AND DEVELOPMENT LIMITED PARTNERSHIP) 27 November 1985 (27.11.85)	(1,2,10,11)
Y	AU,A, 23424/84 (UNITED STATES OF AMERICA, SECRETARY UNITED STATES DEPARTMENT OF COMMERCE) 7 June 1984 (07.06.84)	(1,2,10,11)
Y	Science, Volume 224, issued 1984, April 27 (Washington D.C.), G.L. Smith et al, 'Plasmodium Knowlesi Sporozoite Antigen: Expression by Infectious Recombinant Vaccinia Virus', see pages 397-399.	(1-7,10,11)

Continued

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
16 December 1986 (16.12.86)	(13.01.87) 13 JANUARY 1987
International Searching Authority	**Signature of Authorized Officer**
Australian Patent Office	J.W. Ashman J.W. ASHMAN

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	Modern Approaches Vaccines: Molecular Chemical Basis Virus Virulence Immunogenicity, [Pap.Conf.] 1983, issued 1984 (Cold Spring Harbor, N.Y.), E. Paoletti et al, 'Construction of Live Recombinant Vaccines Using Genetically Engineered Poxvirus', see pages 295-299.	(1,2,10,11)
Y	Immunology Today, Volume 6, No.8, issued 1985, August (Amsterdam), B. Moss, 'Vaccinia Virus Expression Vector: A New Tool for Immunologists', see pages 243-245.	(1-7,10,11)

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons.

1. ☐ Claim numbers .... because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers .... because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers .... because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.